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Seasonal H1N1 Influenza Virus Infection Induces Cross-Protective Pandemic H1N1 Virus Immunity through a CD8-Independent, B Cell-Dependent Mechanism

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During the 2009 H1N1 influenza virus pandemic (pdmH1N1) outbreak, it was found that most individuals lacked antibodies against the new pdmH1N1 virus, and only the elderly showed anti-hemagglutinin (anti-HA) antibodies that were cross-reactive with the new strains. Different studies have demonstrated that prior contact with the virus can confer protection against strains with some degree of dissimilarity; however, this has not been sufficiently explored within the context of a pdmH1N1 virus infection. In this study, we have found that a first infection with the A/Brisbane/59/2007 virus strain confers heterologous protection in ferrets and mice against a subsequent pdmH1N1 (A/Mexico/4108/2009) virus infection through a cross-reactive but non-neutralizing antibody mechanism. Heterologous immunity is abrogated in B cell-deficient mice but maintained in CD8$^{-/-}$ and perforin-1$^{-/-}$ mice. We identified cross-reactive antibodies from A/Brisbane/59/2007 sera that recognize non-HA epitopes in pdmH1N1 virus. Passive serum transfer showed that cross-reactive sH1N1-induced antibodies conferred protection in naive recipient mice during pdmH1N1 virus challenge. The presence or absence of anti-HA antibodies, therefore, is not the sole indicator of the effectiveness of protective cross-reactive antibody immunity. Measurement of additional antibody repertoires targeting the non-HA antigens of influenza virus should be taken into consideration in assessing protection and immunization strategies. We propose that preexisting cross-protective non-HA antibody immunity may have had an overall protective effect during the 2009 pdmH1N1 outbreak, thereby reducing disease severity in human infections.

The novel swine-origin influenza A H1N1 virus was identified as the cause of human respiratory disease in Mexico and the United States in April 2009 (2, 4). This virus was later designated as the pandemic H1N1 2009 virus (pdmH1N1). The emerging virus spread throughout the world and prompted the World Health Organization (WHO) to declare the pandemic alert to level 6 on 11 June 2009 (1). The virus infected millions of people, and at least 14,711 deaths were reported worldwide by 29 January 2010 (5).

Vaccination is a critical intervention intended to diminish the spread of influenza virus and reduce the symptom severity in the infected individuals. Given that pdmH1N1 virus is antigenically and genetically different from previously circulating seasonal H1N1 (sH1N1) influenza virus (15), vaccines that are based on sH1N1 antigens are unlikely to provide cross-reactivity to the pdmH1N1 virus (3). Thus, monovalent pdmH1N1 vaccines have been produced since the emergence of the new influenza virus strains and they are able to achieve seroprotection rates of ca. 85% (8, 28).

Serological analyses performed in prepandemic human serum samples showed that cross-neutralizing antibodies against pdmH1N1 virus were present in the elderly population but not in children and young adults (18, 21, 32). These antibodies are possibly a consequence of previous exposure to older viruses that were antigenically related to pdmH1N1 virus (31, 38), and their presence may explain the overall low symptom severity that was observed among the elderly during the 2009 pandemic (7, 32). Furthermore, several studies in animal models have demonstrated that a prior infection with sH1N1 virus is able to provide substantial protection against pdmH1N1 virus infection (12, 13, 23, 27); cross-reactive CD8 and CD4 T cell responses against pdmH1N1 viruses were detected, indicating that a substantial fraction of the T cell epitopes is conserved between sH1N1 and pdmH1N1 viruses (39, 40). Also, B cell responses can provide extensive cross-protection against drifted influenza virus strains (41).

In the present study, we have found that a first infection with sH1N1 A/Brisbane/59/2007 virus confers heterologous protection in ferrets and mice against a subsequent challenge with pdmH1N1 A/Mexico/4108/2009 virus through a cross-reactive but non-neutralizing antibody mechanism. Heterologous immunity is heavily diminished in B cell-deficient mice but maintained in CD8$^{-/-}$ and perforin-1$^{-/-}$ (Prf1$^{-/-}$) mice. We identified cross-reactive antibodies from A/Brisbane/59/2007 sera that recognize

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non-hemagglutinin (HA) epitopes from the pdmH1N1 virus. Moreover, passive transfer of cross-reactive antibodies induced by sH1N1 virus infection provided substantial protection against pdmH1N1 virus challenge in naive recipient mice. Our study indicates that sH1N1 virus primary infection induced preexisting non-HA antibodies and/or memory B cells, and they are essential for providing cross-protective immunity against a subsequent pdmH1N1 virus challenge in animal models. Assuming that human immune responses will show an analogous behavior during a heterologous reinfection, we propose that previous encounters with sH1N1 virus exerted an overall protective effect in the human population during the 2009 pandemic.

MATERIALS AND METHODS

Animals and viruses. Male ferrets 4 to 6 months old were purchased from Marshall Bioresources (New York, NY), and they were proven to be seronegative against different influenza virus strains. Pathogen-free C57BL/6, CD8 T cell-deficient strain Cd8a^{tm1Mak}/J (CD8$^{-/-}$) mice, immunoglobulin μ heavy-chain mutant strain Ighm^{tm1Cgn}/J (μMT) mice, and perforin-1-deficient strain Prf1^{tm1sdz}/J (Prf1$^{-/-}$) mice (8 to 10 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, ME). Experiments with ferrets and mice were conducted at the Animal Resources Centre of University Health Network (UHN; Toronto, Canada) under BSL-2+ conditions and in accordance with the Canadian Council of Animal Care guidelines. The animal use protocols were approved by the Animal Care Committee of UHN. All viruses were obtained from U.S. Centers for Disease Control (Atlanta, GA) and grown in the allantoic cavity of 10-day-old embryonated chicken eggs. The virus titer, expressed as the 50% egg infective dose (EID_{50})/ml, was calculated by serially titrating the virus on chicken eggs according to the Reed-Muench method. The A/California/07/2009 virion was split by Triton X-100 and inactivated by formaldehyde at 4°C overnight. The split viral components were then collected by high-speed centrifugation and diluted in phosphate-buffered saline (PBS). The resulting split virus contains all of the structural proteins such as HA, neuraminidase (NA), nucleoprotein (NP), and matrix protein (M). Specifically, we determined the presence of HA and NP by enzyme-linked immunosorbent assay (ELISA).

Seasonal H1N1 virus (sH1N1) primary infection and pandemic H1N1 virus (pdmH1N1) secondary challenge. To evaluate the cross-protection provided by human seasonal H1N1 virus primary infection, naive ferrets, C57BL/J mice and mutant mice were infected by 10^6 EID_{50} of A/Brisbane/59/2007 intranasally (i.n.). The clinical signs were monitored daily postinfection. Ferrets and mice were rechallenged 4 to 5 weeks after the first infection with sH1N1; a similar period of time between the first and the second infection has been used in other studies (17, 25, 27, 34).

Ferrets were challenged by pdmH1N1 virus at week 4 postvaccination or primary infection. Animals were moved at least 4 days prior to infection to the BSL-2 animal holding area, where they were housed in cages contained in Bioclean portable laminar flow clean room enclosures (Lab Products, Seaford, DE). Prior to infection, baseline temperatures and weights were measured once daily for at least 3 days. Ferrets were anesthetized with 5% isoflurane and infected i.n. with a total of 1 ml of 10^6 EID_{50} of the A/Mexico/4108/2009 or A/California/07/2009 virus strain per ml in PBS delivered to the nostrils. Temperatures were measured daily using a subcutaneous implantable temperature transponder (BioMedic Data Systems, Inc., Seaford, DE). Clinical signs in terms of temperature change, weight loss, nasal discharge, and inactivity were recorded daily postinfection as described previously (14).

In our mouse study, seasonal virus-infected wild-type (WT) C57BL/6 mice, mutant mice, and uninfected WT mice were challenged with 10^5 EID_{50} of A/Mexico/4108/2009 virus at week 5 after primary infection. The body weights were monitored once daily, and mice were humanely euthanized when losing 20% or more of their original weights.

Viral load assay in nasal wash samples and lung tissue. Ferret nasal wash samples and mouse lung tissue were collected after pdmH1N1 virus infection. Dissected lung tissue was homogenized in Dulbecco modified Eagle medium by 1:10 (wt/vol) and then centrifuged at 3,000 rpm for 15 min to collect the supernatant. The viral load in nasal wash and lung homogenate samples was determined by using a 6-day MDCK cell culture-based assay as described in our previous study (14). The virus titer, expressed as the 50% tissue culture infective dose ($TCID_{50}$)/ml from each nasal wash sample, was calculated according to the Reed-Muench method.

Histopathology. Lung tissue collected from the infected animals was perfused and fixed in 10% formalin and then paraffin embedded. Tissue section was placed on a positive charged slide and stained with hematoxylin and eosin (H&E) for histopathologic examination.

Hemagglutination inhibition (HI) test. Serum samples were treated with receptor-destroying enzyme (RDE) at 37°C overnight. Fresh turkey red blood cells (TRBC) were washed and diluted in PBS to a concentration of 0.5% (vol/vol). The sera were serially diluted in PBS in 96-well V-bottom cell culture plates. The serially diluted sera were incubated with 25 μl (8 HA U/50 μl) virus for 15 min. Then, 50 μl of 0.5% TRBC was added, and the plates were incubated at room temperature for 30 min. The hemagglutination inhibition (HI) titer was the reciprocal of the highest serum dilution to completely prevent agglutination.

MN assay. The serum-neutralizing antibodies were determined by using the pdmH1N1 A/Mexico/4108/2009 (H1N1) virus by microneutralization (MN) assay described previously (14). Briefly, the $TCID_{50}$ of each virus was determined by titration in MDCK cells under biosafety level 2 conditions. The serially 2-fold-diluted RDE-treated serum at a starting dilution of 1:10 was tested for neutralizing 100 $TCID_{50}$ of each virus/50 μl in an MDCK cell monolayer. The cytopathic effect was determined after incubation for 20 h.

ELISA. Serum and lung homogenate samples were assessed for antibody level by ELISA. Briefly, ELISA plates were coated with inactivated split pandemic H1N1 A/California/07/2009 virus (5 μg of HA/ml), recombinant H1N1 virus (seasonal H1N1 A/Bribane/59/2007 virus, pandemic H1N1 virus A/California/07/2009; Sino Biological, Inc., China) HA (5 μg/ml), or recombinant H1N1 virus (A/Puerto Rico/8/34) NP (5 μg/ml) overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 (T-PBS) and blocked with 1% bovine serum albumin for 1 h at 37°C. Antigen-coated plates were washed with T-PBS and incubated with 1:1,000-diluted samples overnight at 4°C. After washing with T-PBS, the plates were incubated with goat anti-mouse immunoglobulin (IgG, IgG1, IgG2a, and IgA) horseradish peroxidase (HRP) conjugates (Santa Cruz) in a 1:2,000 dilution for 1 h at 37°C. The reaction was developed by *o*-phenylenediamine, and the optical density was read by using excitation and emission wavelengths of 490 and 570 nm, respectively. The IgG1 and IgG2a standard curves generated by plotting the density readout, and the serially diluted amounts of protein standard were used for calculating the concentration of samples.

Western blotting. The boiled recombinant sH1N1 HA, pdmH1N1 HA, conservative H1N1 NP, and pdmH1N1 M1 (A/California/04/2009; Immune Technology Corp.) protein was equally loaded (0.5 μg/lane) and subjected to SDS–12% PAGE. The gels were subsequently transferred onto nitrocellulose membrane for 1 h at 100 V. The membrane was blocked with 5% milk–T-PBS for 45 min at room temperature and then incubated with 1:1,000-diluted serum samples (developed by the Kelvin laboratory) overnight at 4°C, followed by HRP-conjugated goat anti-mouse secondary antibody incubation. The bands were developed with a BM chemiluminescence Western blotting substrate kit (Roche Diagnostics, Laval, Quebec, Canada) and visualized using Kodak film developer.

Enrichment of lymphocytes from the lung and spleen. Methods lymphocyte enrichment have been described in previously published studies (29, 30). In brief, after lung tissue was collected, it was cut into small pieces and then mechanically homogenized into RPMI 1640 medium. The suspension was passed though 40-μm-pore-size nylon mesh filter to remove

major tissue fragments and fibroblasts. The filtered clear suspension was placed onto a mouse Lympholyte (Cedarlane) layer and centrifuged at 1,600 rpm for 20 min. The lymphocyte band was collected and washed three times with PBS before use.

Lymphocytes from spleen were also enriched in a mechanical way. The filtered cell suspension was treated with red blood cell lysate buffer and then washed three times with PBS to generate a single cell suspension.

Flow cytometry analysis. Lung lymphocytes and splenocytes isolated after pdmH1N1 virus infection were analyzed for T cell and B cell activation and/or memory phenotype by staining with mouse anti-CD3, anti-CD8, anti-CD44, anti-CD19, anti-CD69, and anti-CD27 antibodies (eBioscience). After staining, the samples were run through a BD FACSCalibur (BD Biosciences), and the data were analyzed by using FlowJo software (TreeStar).

Lymphocyte *in vitro* stimulation and intracellular staining. For gamma interferon (IFN-γ) intracellular staining, isolated lung lymphocytes and splenocytes at a concentration of 10^6 cells/ml were cultured with live A/Mexico/4108/2009 or A/Brisbane/59/2007 virus in complete RPMI 1640 medium at 37°C and 5% CO_2 for 10 h. To minimize the indirect manner of IFN-γ stimulation by live virus, a low multiplicity of infection (MOI) of 0.1 was used for the *ex vivo* cell coculture. After stimulation, cells were washed by 1× PBS and stained with mouse anti-CD3 and anti-CD8 antibody. After surface staining, the cells were fixed and permeabilized before staining with IFN-γ antibody. Samples were run on a BD FACSCalibur (BD Biosciences), and the data were analyzed by using FlowJo software.

Analysis and comparison of B cell epitope sequences. Predicted B cell linear epitopes toward HA, NA, NP, and matrix protein 1 (M1) of A/Brisbane/59/2007 and A/Mexico/4108/2009 were generated by using a Kolaskar and Tongaonkar antigenicity scale (24) from the IEDB analysis resources (http://tools.immuneepitope.org/main/). The similarity of predicted B cell epitope sequences of influenza virus antigens from the two influenza viruses was evaluated by using the IEBD epitope conservancy analysis tool.

Serum passive transfer. Ferret serum was collected 14 days after infection with A/Brisbane/59/2007 or A/Mexico/4108/2009 virus; the presence of moderate to high HI titers against the same virus strain was confirmed to assure the validity of the infections. Also, serum collected from naive ferrets was used as a negative control. Naive mice received 200 μl of serum by intraperitoneal injection daily from day −3 to day 0 before infection with 10^5 EID_{50} of A/Mexico/4108/2009 virus. After infection, the mice were monitored daily for weight loss and lethality.

Statistics. The Student *t* test and one-way analysis of variance were used for statistical analysis of the results. A log-rank (Mantel-Cox) test was used to analyze the difference among survival curves. A P value of ≤ 0.05 was considered significant.

RESULTS AND DISCUSSION

In order to investigate whether a prior infection with sH1N1 virus is able to induce protection against a secondary challenge with pdmH1N1, ferrets were infected with 10^6 EID_{50} of sH1N1 A/Brisbane/59/2007 (Brisbane/59) virus and subsequently challenged with 10^6 EID_{50} of pdmH1N1 A/Mexico/4108 virus (Mexico/4108). Ferrets infected with sH1N1 virus only developed mild clinical symptoms. Ferrets showed increased temperature only at day 2 postinfection and a maximum weight loss of 3.9%; the clinical findings were very similar to those shown in a previous work published by our group, which included a more extensive pathological evaluation of ferrets infected with A/Brisbane/59/2007 (35). In the present study, serological assays demonstrated that Brisbane/59 virus primary-infected animals had neither cross-reactive HI titers nor cross-neutralizing antibodies against pdmH1N1 virus before challenge (Fig. 1A and B). Nonetheless, we found that the primary infection with sH1N1 virus in ferrets conferred robust protection against a secondary infection with either pdmH1N1 Mexico/4108 or A/California/07/2009 virus (Fig. 1C and D; see Fig. S1 in the supplemental material). The immunity induced by Brisbane/59 virus infection dramatically reduced the disease severity in ferrets, in terms of weight loss, temperature change, viral load, and lung pathology (Fig. 1C to E; see Fig. S2A in the supplemental material). Furthermore, we demonstrated the same protective effect in C57BL/6 mice. Mice infected with 10^6 EID_{50} of Brisbane/59 showed a slight weight loss of maximal 3.1% without lethality and, during a second challenge with 10^5 EID_{50} of Mexico/4108 virus, a significant reduction in the disease severity was observed. sH1N1 virus primary-infected mice showed a peak of 12% weight loss on day 4 postinfection with no mortality (Fig. 2A and B), while the pdmH1N1-only infection group showed 95% mortality, significantly higher virus titers in lungs, and also extensive lung pathology characterized by acute alveolitis and bronchiolitis (Fig. 2A to C; see Fig. S2B in the supplemental material). Taken together, these results demonstrate that prior infection with sH1N1 virus confers protection against pdmH1N1 virus. On the other hand, we found that immunization with a seasonal vaccine was of little value in providing cross-protection against pdmH1N1 virus (see Fig. S4 in the supplemental material), confirming previous results (12).

We next evaluated the antibody responses that were generated after influenza virus infection in mice. First, HI titers showed that antibodies induced by Brisbane/59 infection were not cross-reactive with Mexico/4108 (Fig. 3A). The Western blot analysis also showed that anti-HA IgG1 and IgG2a antibodies are influenza virus strain specific; Brisbane/59 virus-infected animals showed only anti-Brisbane HA antibodies, whereas Mexico/4108-infected animals showed only anti-pdmH1N1 HA antibodies (Fig. 3B). Although anti-Brisbane HA antibodies were highly increased in Brisbane/59 virus primary-infected mice at day 4 after heterologous virus challenge (Fig. 3B), no antibodies against pdmH1N1 HA were seen at this time point (Fig. 3A and B). These results confirm that neutralizing antibodies against the external influenza virus antigens HA are highly specific against closely related influenza virus strains (10).

Antibodies targeting the internal viral proteins also represent a major component of the antibody repertoires (16). Given that different putative B cell epitopes of the internal proteins are highly conserved between the Brisbane/59 and Mexico/4108 viruses (see Table S1 in the supplemental material), we analyzed the presence of cross-reactive antibodies against split pdmH1N1 virus by ELISA. We found that cross-reactive IgG1 and IgG2a antibodies against split pdmH1N1 virus were elevated in Brisbane/59 virus-infected mice (Fig. 3C), and it was after homologous infection that higher antibody titers were observed (Fig. 3C). Moreover, IgG1 antibodies against split pdmH1N1 virus in Brisbane/59 virus primary-infected mice were significantly increased at day 4 after pdmH1N1 virus secondary infection (Fig. 3C). On the other hand, the viral NP also showed high antigenic similarity between the sH1N1 and pdmH1N1 viruses (see Table S1 in the supplemental material); detection of anti-NP antibodies in serum was carried out by using recombinant NP from PR8 virus, which shows 91% similarity with NP from pdmH1N1. ELISA and Western blot analyses showed that anti-NP IgG1 and IgG2a antibodies were induced during the infection with either Brisbane/59 or Mexico/4108 virus (Fig. 3D and E), and they were preferentially stimulated toward to the IgG1 isotype (Fig. 3D and E). Next, we examined the

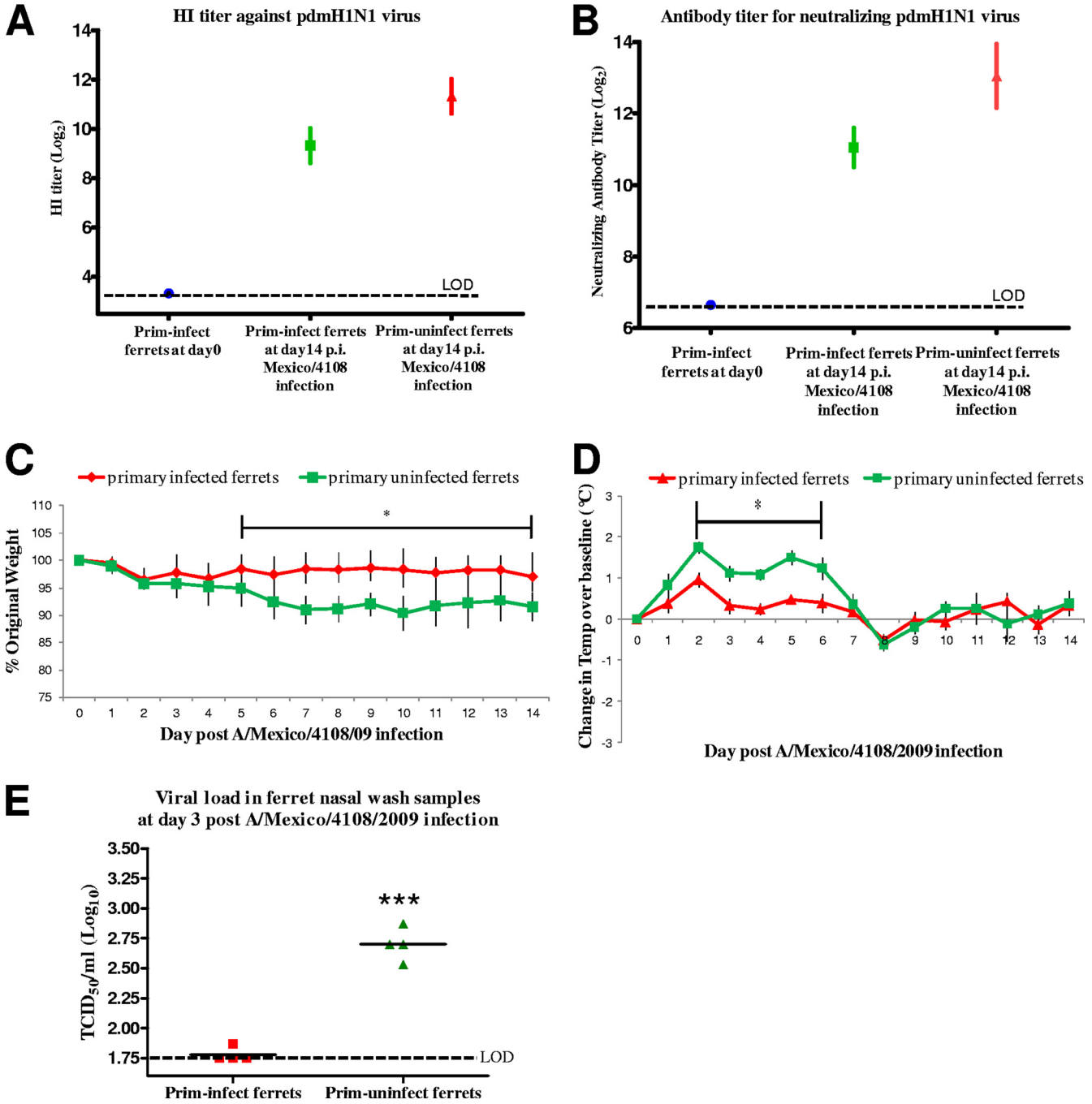

FIG 1 Evaluation of antibody response, clinical signs, and virus titer for seasonal H1N1 virus primary-infected ferrets following secondary infection with A/Mexico/4108/2009 virus. Ferrets were primary infected with 10^6 EID_{50} of A/Brisbane/59/2007 virus or left uninfected as control. Purified serum was treated by RDE at 37°C overnight before HI and microneutralization (MN) assays. (A) Antibody titers against pdmH1N1 virus in A/Brisbane/59/2007 virus-infected ferret (Bris/59+Mex/4108) serum samples, as well as uninfected ferret serum samples ($n = 4$) collected at days 0 and 14 after pdmH1N1 virus secondary infection were measured by HI assay. (B) Neutralizing antibody titers for blocking pdmH1N1 virus were measured by MN assay in the same serum samples investigated by HI test. (C and D) Weight loss (C) and body temperature (D) changes were determined daily for the respective groups ($n = 8$/group) after the secondary infection with 10^6 EID_{50} doses of A/Mexico/4108/2009 virus. The data points represent mean values, and error bars demonstrate standard errors of the mean. (E) The viral load in ferret nasal wash samples ($n = 4$) collected at day 3 after pdmH1N1 virus infection was determined. Horizontal bars demonstrate mean values. *, $P \leq 0.05$; ***, $P \leq 0.001$; LOD, limit of detection.

presence of antibodies targeted against the M1 antigen by using recombinant M1 protein from pdmH1N1 A/California/04/2009 virus. Serum from Brisbane/59 virus-infected mice showed less than one-half of the band intensity compared to serum from pdmH1N1 virus-infected mice, suggesting that anti-M1 IgG antibodies are only partially cross-reactive between sH1N1 and pdmH1N1 viruses (Fig. 3F). Taken together, the presence of antibodies reactive against split pdmH1N1 virus, viral NP, and also

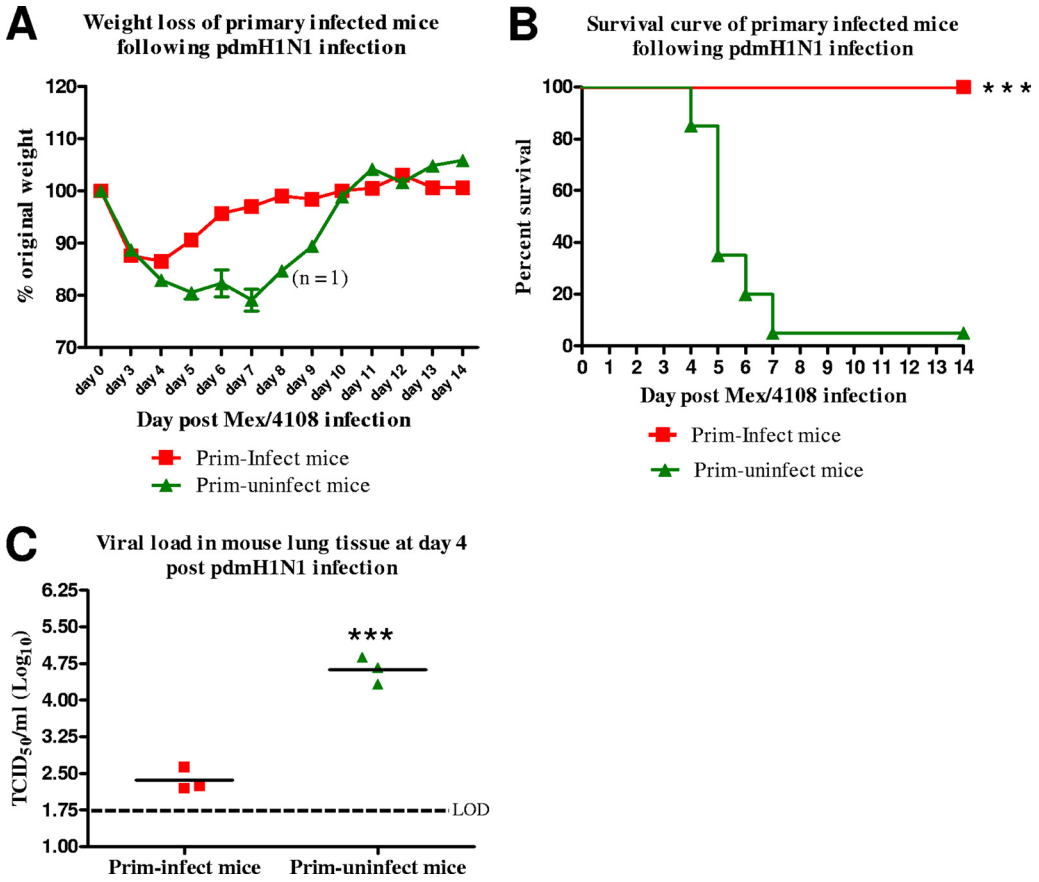

FIG 2 Assessment of infection outcome and virus titer for seasonal H1N1 virus primary-infected mice after secondary infection with A/Mexico/4108/2009 virus. C57BL/6 mice were primary infected with 10^6 EID_{50} of A/Brisbane/59/2007 virus or left uninfected as control. Five weeks later, the mice were rechallenged with 10^5 EID_{50} of A/Mexico/4108/2009 virus. (A and B) The weight loss (A) and lethality (B) of A/Brisbane/59/2007 virus-infected C57BL/6 mice and uninfected C57BL/6 mice ($n = 20$/group) were monitored after pdmH1N1 virus secondary infection. Comparison of survival curves was analyzed by a log-rank (Mantel-Cox) test. Only one mouse ($n = 1$) survived among the primary uninfected group mice after pdmH1N1 virus infection. (C) The viral load in mice lung tissue samples ($n = 3$) collected at day 4 after pdmH1N1 virus challenge was determined. Horizontal bars demonstrate mean values. ***, $P \leq 0.001$; LOD, limit of detection in viral load assay.

viral M1 to a lesser extent prove that the primary infection with Brisbane/59 virus induces the production of cross-reactive antibodies against the conservative viral antigens; these cross-reactive antibodies may partially account for the *in vivo* protection that is induced by a previous exposure to sH1N1 virus (Fig. 2). Although HI titers and levels of neutralizing antibodies are still useful indicators of specific immunity against influenza, the measurement of the cross-reactive antibodies against the internal viral antigens may provide a global picture of the overall cross-protective immunity during a heterologous virus infection.

The levels of specific antibodies in the infected tissues correlate well with the level of protection against secondary infections (20). Upon a heterologous infection, we were able to detect the presence of cross-reactive antibodies in the lungs, showing an expression pattern similar to the one found in the sera (see Fig. S3 in the supplemental material), except for the higher levels of IgA antibodies that were detected at the site of infection. These results are consistent with a role that local antibody responses play during the resolution of influenza virus infections (20).

Apart from inducing high titers of cross-reactive antibodies, prior infection with influenza Brisbane/59 virus also influenced the cellular response kinetics during the subsequent heterologous infection with Mexico/4108 virus. Cross-reactive memory/effector $CD8^+$ $CD44^{high}$ T cells, as well as $CD8^+$ IFN-γ^+ T cells, were significantly increased in enriched lung lymphocytes (Fig. 4A and B) and splenocytes (data not shown) of Brisbane/59 virus primary-infected mice during pdmH1N1 virus rechallenge. We also observed significantly increased percentages of activated B cells ($CD19^+$ $CD69^+$) and memory B cells ($CD19^+$ $CD27^+$) in the lungs of primary-infected mice which showed limited viral shedding at day 4 after pdmH1N1 virus infection (Fig. 4C and D; Fig. 2C), nevertheless, it is possible that the higher levels of B cells observed during the reinfection may be due partially to remaining cells that were induced during the primary infection. Taken collectively, these results indicate that sH1N1 virus primary infection induces cross-reactive memory T cells and B cells, resulting in enhanced local immune responses during the second infection.

In order to further evaluate the importance of B cell and cytotoxic T cell responses in heterotypic immunity, B cell-deficient (μMT), CD8-deficient, and Prf1-deficient mice were infected first with Brisbane/59 virus and 5 weeks later challenged with Mexico/4108 virus. As mentioned above, primary infection with Brisbane/59 virus conferred immunological protection in WT mice during heterologous reinfection with Mexico/4108 virus (Fig. 2).

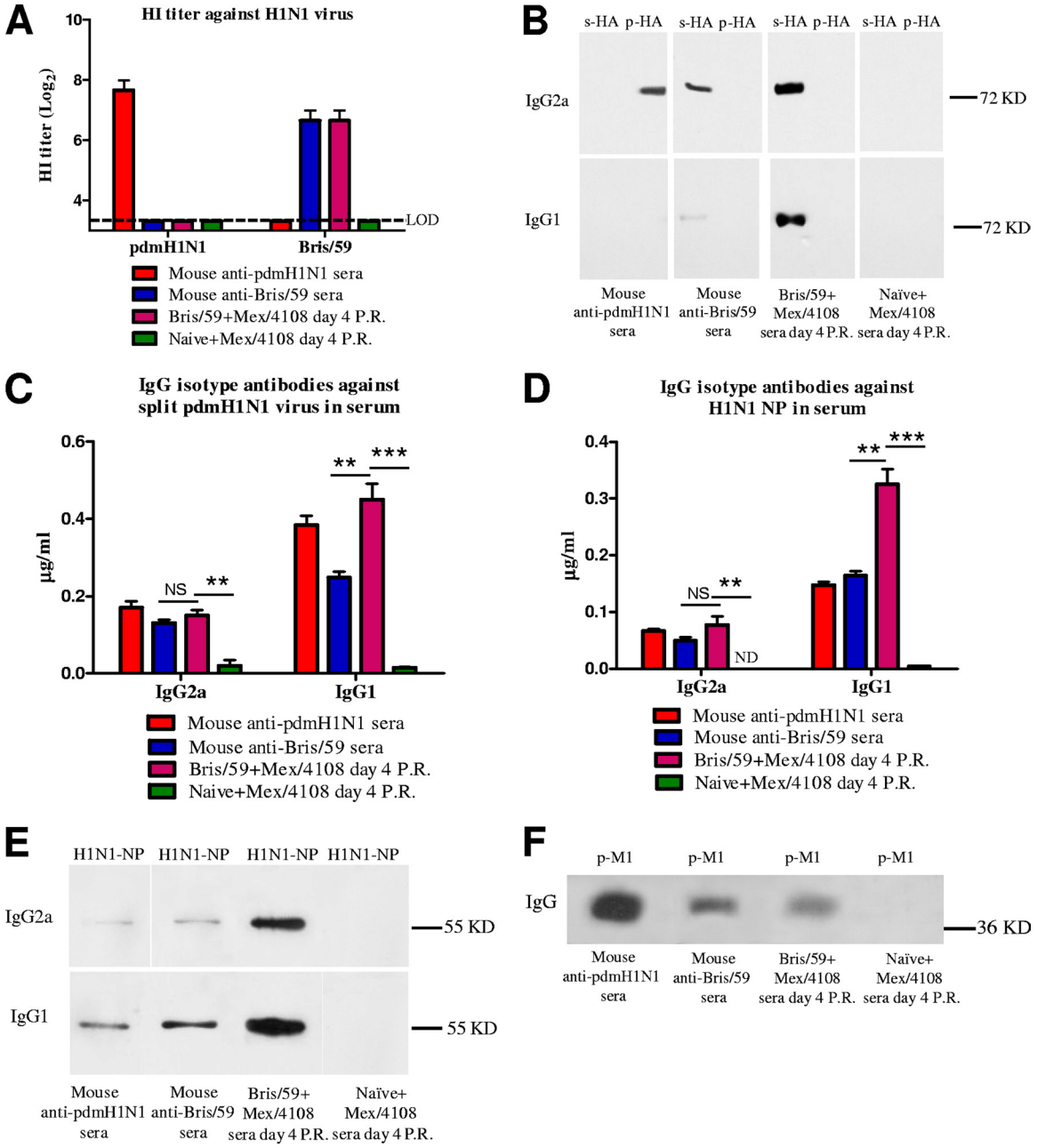

FIG 3 Evaluation of cross-reactive activity of antibodies induced by human seasonal H1N1 virus in mice. Mouse anti-pdmH1N1 sera (collected at day 14 after a sublethal infection with A/Mexico/4108/2009), anti-Brisbane/59 sera, sH1N1 virus primary-infected mice sera collected at day 4 after pdmH1N1 virus challenge (Bris/59+Mex/4108 day 4 P.R.), and primary uninfected mice sera collected at day 4 after pdmH1N1 virus challenge (naive+Mex/4108 day 4 P.R.) were assessed for antibody responses. (A) HI test was performed for assessing Brisbane/59 or pdmH1N1 virus-specific HI titers. (B) Mice sera were evaluated for cross-reactive IgG1 and IgG2a antibodies against recombinant sH1N1 HA (s-HA) and pdmH1N1 HA (p-HA) by Western blotting. (C and D) Cross-reactive IgG1 and IgG2a antibodies against (C) split pdmH1N1 and (D) recombinant H1N1 NP (A/Puerto Rico/8/34) were analyzed by ELISA. (E) Isotype IgG antibodies against H1N1 NP in mice sera were evaluated by Western blotting. (F) Total IgG antibodies against recombinant pdmH1N1 (A/California/04/2009) M1 (p-M1) protein in mice sera were evaluated by Western blotting. The average relative absorbance density from three individual samples was read by using excitation and emission wavelengths of 490 and 570 nm, respectively. The concentrations of IgG isotype antibodies were then calculated based on the linear standard curve. The result of a representative sample is shown in the Western blot. ND, not detected; NS, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$, ***; $P \leq 0.001$; LOD, limit of detection in HI assay.

However, B cell-deficient mice showed 90% lethality, as well as a high level of lung virus titers similar to those found in the group of naive mice infected with Mexico/4108 virus (Fig. 5A). This result indicates that the protection induced by Brisbane/59 virus primary infection significantly relies on the presence of B lymphocytes. Interestingly, CD8-deficient and WT mice infected with sH1N1 virus showed similar patterns of weight loss during Mexico/4108 virus reinfection (data not shown), and 100% of the CD8-

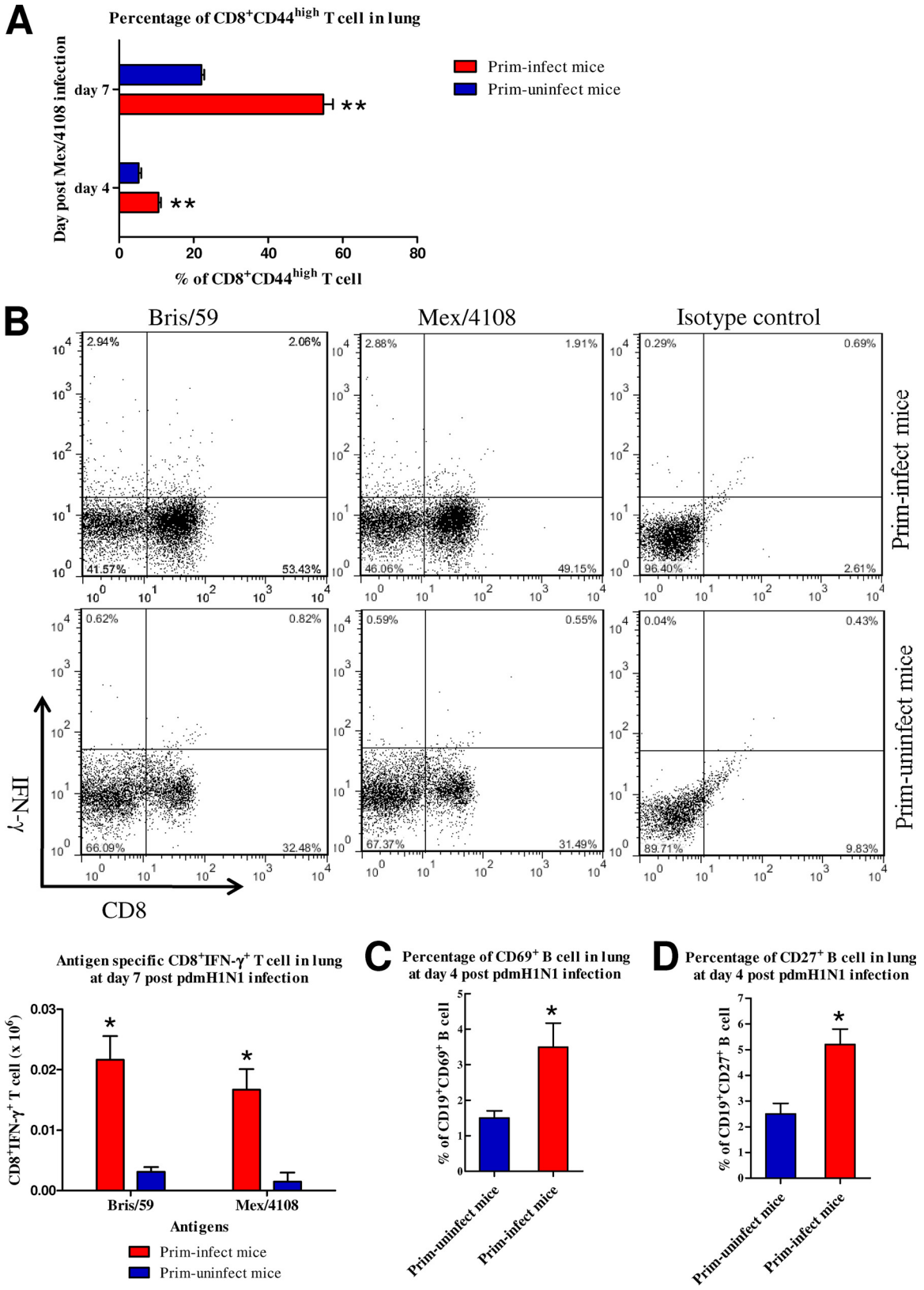

FIG 4 Evaluation of cross-responsive CD8 T cells and B cells in the lungs of seasonal H1N1 virus primary-infected mice after pdmH1N1 virus rechallenge. Lymphocytes were enriched from seasonal virus primary-infected and uninfected mouse lung tissue (n = 3/group) at days 4 and 7 after pdmH1N1 virus secondary infection. (A) Analyses of the memory/effector phenotype CD8 T cell ($CD8^+$ $CD44^{high}$) response in mouse lung lymphocytes by flow cytometry. (B) Antigen-specific $CD8^+$ IFN-γ^+ T cell in mouse lung lymphocytes was determined by flow cytometry (dot plot figures of a representative sample are shown in upper panel). The total number of antigen-specific $CD8^+$ IFN-γ^+ T cells in mouse lung lymphocytes is shown in the lower panel. (C and D) Percentages of activated B cells ($CD19^+$ $CD69^+$) (C) and memory phenotype B cells ($CD19^+$ $CD27^+$) (D) in lungs collected from primary-infected and uninfected mice (n = 3/group) at day 4 after pdmH1N1 virus secondary infection were measured by flow cytometry. *, $P \leq 0.05$; **, $P \leq 0.01$.

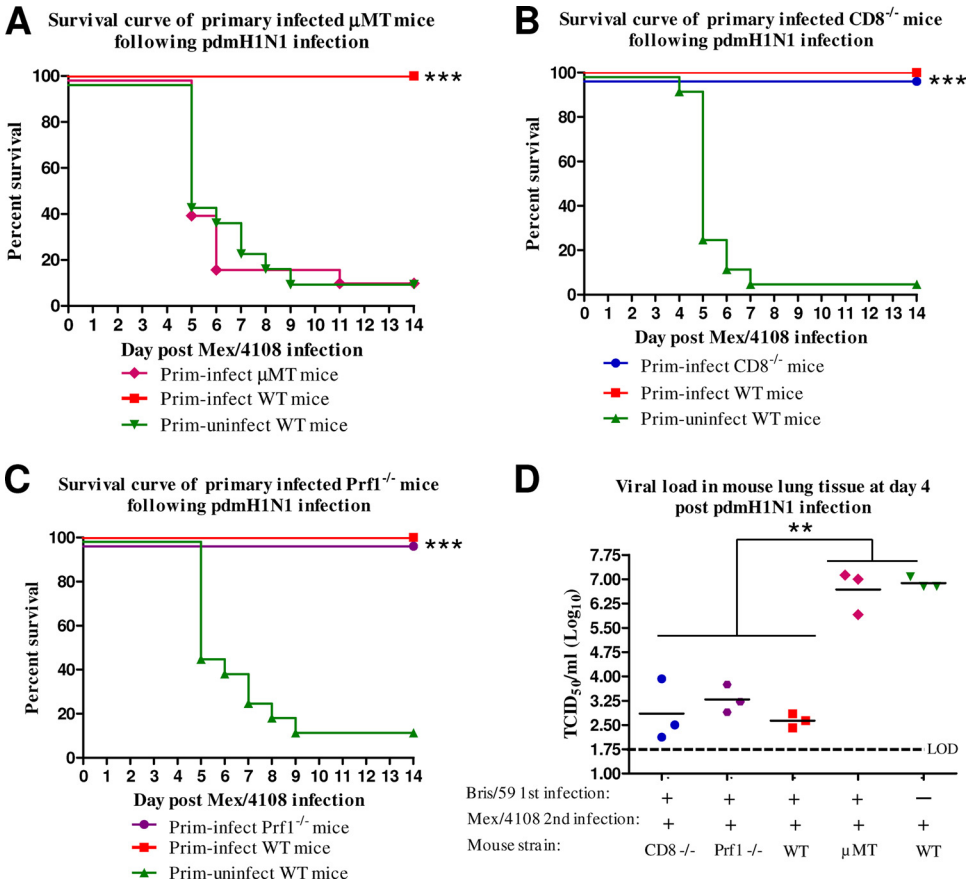

FIG 5 Mortality and virus titer assessment of primary-infected mutant mice after pdmH1N1 virus rechallenge. Mice were primary infected with 10^6 EID_{50} of A/Brisbane/50/2007 virus and 5 weeks later challenged with 10^5 EID_{50} of A/Mexico/4108 virus. The lethalities of primary-infected B cell-deficient (μMT) mice (A), primary-infected CD8 T cell-deficient ($CD8^{-/-}$) mice (B), and primary-infected perforin-1-deficient ($Prf1^{-/-}$) mice (C) ($n = 20$/group) were monitored after pdmH1N1 virus secondary infection. Primary-infected WT C57BL/6 mice and primary-uninfected WT C57BL/6 mice ($n = 20$/group) were used as positive and negative controls, respectively, in the study. Comparison of survival curves was analyzed by log-rank (Mantel-Cox) test. (D) Virus titer in lungs ($n = 3$) collected at day 4 after pdmH1N1 virus secondary infection. The virus titer was calculated by the Reed-Muench method and is expressed as the $TCID_{50}$/ml. Horizontal bars demonstrate mean value. **, $P \leq 0.01$; ***, $P \leq 0.001$; LOD, limit of detection in viral load assay.

deficient mice survived pdmH1N1 virus rechallenge (Fig. 5B). Therefore, the lack of CD8 T cells is not detrimental for the induction of cross-protective immunity (Fig. 5D). Moreover, we found that cross-protective immunity was not affected by the impairment of granzyme/perforin-mediated cytotoxic response. During secondary infection with Mexico/4108 virus, both Prf1-deficient and WT mice primary infected with Brisbane/59 virus showed 100% survival and had similar levels of viral shedding in lungs (Fig. 5C and D).

In order to assess the capacity of preexisting antibodies to confer protection during a heterologous rechallenge, serum from naive ferrets or previously infected with sH1N1 or pdmH1N1 virus was passively transferred to naive mice from day −3 to day 0 before pdmH1N1 virus infection. As expected, the sera from naive ferrets was unable to alter the course of the infection in mice (Table 1). Ferret anti-Mexico/4108 virus serum was able to confer protection in naive mice against pdmH1N1 virus infection without showing any significant weight loss (Table 1). Interestingly, ferret anti-Brisbane/59 serum, which showed no cross-reactive HI titers against pdmH1N1 virus, was capable of significantly reducing weight loss and resulted in the rescue of 90% of the naive recipient mice from pdmH1N1 virus infection (Table 1). These results confirm that high levels of neutralizing antibodies are required to achieve the maximum level of protection but, at the same time, the cross-reactive non-HA, and possibly non-neutralizing antibodies can also play an important protective role in the absence of HI titers (25).

TABLE 1 Outcome of pdmH1N1 virus infection in mice treated with ferret anti-influenza virus serum[a]

Donor ferret anti-influenza virus serum	HI titer against:		% Wt loss (day)[b] of treated mice	Lethality (%) in treated mice[c]
	sH1N1 virus in donor serum	pdmH1N1 virus in donor serum		
Anti-Mex/4108	<10	1,280	0.6 (6)	0*
Anti-Bris/59	1,280	<10	8.2 (7)	10*
Naive	<10	<10	22.9 (9)	100

[a] Ferret anti-influenza virus serum was intraperitoneally injected into naive mice (10 mice/group) daily from day −3 to day 0 before infection with 10^5 EID_{50} of the A/Mexico/4108/2009 virus strain.
[b] That is, the day of peak weight loss postchallenge from the original weight.
[c] *, $P < 0.001$ (log-rank sum test compared to the group treated with serum from a naive ferret).

Although many studies have tried to establish the relative contribution of antibodies and cytotoxic responses in the immunity against influenza virus infection, the answer is still not clear-cut, and the experimental variables of each study need to be carefully considered. In the context of heterologous immunity, our results showed that efficient antibody production was required to achieve protection in mice, this protection was not ameliorated in CD8-deficient or perforin-deficient mice, and passive transfer serum was able to confer heterologous protection. An analogous scenario was observed by Nguyen et al. in the context of heterosubtypic immunity (first H3N1virus and later H1N1 virus), where protection was ameliorated in antibody-deficient mice but not in CD8-deficient mice (33). On the other hand, Guo et al. found that heterosubtypic immunity was strongly dependent on the presence of CD8 cells in their experimental model in which passive serum transfer was unable to confer protection (17). While keeping in mind the contribution of influenza virus-specific cytotoxic CD8 cells during influenza virus rechallenge (19), we hypothesize that cytotoxic CD8 cells may be dispensable in scenarios where the antibody response meets certain levels of strength and specificity and is able to limit the infection on its own. It is reasonable to think that vaccines induce a less efficient antibody response compared to a proper influenza virus infection, and this would explain why vaccine-induced immunity relies more heavily on the cytotoxic response (13, 36).

Influenza virus infection induces the production of antibodies against most of the 10 viral proteins (37); however, it is the subset of neutralizing anti-HA antibodies that exerts the largest contribution to block the virus entry to the cells. Due to the significant antigenic dissimilarity between the HA protein from seasonal and pandemic H1N1, the neutralizing antibodies induced by the seasonal vaccination or prior infection are unable to accomplish their mission against pdmH1N1 virus (22, 27). The HA2 region of the HA protein possesses a relatively high degree of similarity among different influenza virus strains, and this fact has caused an increasing interest in HA2-based peptides for vaccine development (42). However, anti-HA2 antibodies represent a relatively small fraction of the neutralizing antibodies induced by seasonal vaccines (9). Moreover, a critical antigenic determinant on position 89 of the HA2 region from sH1N1 virus, which had been present in the circulating influenza virus strains for decades, is no longer present in pandemic H1N1 virus, and this has been suggested to be one of the causes of the antigenic escape of the new variants (43). Considering the results from these studies, it seems that the biological relevance of HA2-targeted antibodies in the context of a heterologous infection or vaccination is still unclear, and this requires further study. Other approaches toward the design of "universal influenza vaccines" that are effective against different influenza virus subtypes are based on antigens derived from NP or M2e (6, 11, 26). Unlike anti-HA neutralizing antibodies that act through direct blockage of the virus, the anti-NP and anti-M2e antibody-mediated heterosubtypic immunity requires FcRs that are involved in phagocytosis and/or antibody-dependent cell-mediated cytotoxicity (11, 25); therefore, it is likely that these antibodies relying on a receptor-dependent pathway also play a relevant role in the heterologous protection found in our experimental model.

We have provided here solid experimental evidence of the protective immunity that sH1N1 virus induces against a later challenge with pdmH1N1 virus in both ferrets and mice. If humans behave immunologically in a similar fashion, then prior infection with seasonal H1N1 virus likely resulted in attenuated disease severity. This may partially explain the modest number of severe cases reported during the pandemic phase of the pdmH1N1 virus outbreak. Our results favor an immunological model wherein B cells generate cross-reactive non-HA and non-neutralizing antibodies that confer protection against pdmH1N1 virus. We also explored the role of seasonal vaccines in providing cross-protection and, like other researchers, found that vaccination was of little value.

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REFERENCES

1. **Anonymous.** 2009. New influenza A (H1N1) virus: global epidemiological situation, June 2009. Wkly. Epidemiol. Rec. **84**:249–257.
2. **Anonymous.** 2009. Outbreak of swine-origin influenza A (H1N1) virus infection: Mexico, March-April 2009. MMWR Morb. Mortal. Wkly. Rep. **58**:467–470.
3. **Anonymous.** 2009. Serum cross-reactive antibody response to a novel influenza A (H1N1) virus after vaccination with seasonal influenza vaccine. MMWR Morb. Mortal. Wkly. Rep. **58**:521–524.
4. **Anonymous.** 2009. Update: swine influenza A (H1N1) infections: California and Texas, April 2009. MMWR Morb. Mortal. Wkly. Rep. **58**:435–437.
5. **Anonymous.** 2010. Pandemic (H1N1) 2009: update 85. Document 2010.2-2-2010. World Health Organization, Geneva, Switzerland. http://www.who.int/csr/don/2010_01_29/en/index.html.
6. **Carragher DM, Kaminski DA, Moquin A, Hartson L, Randall TD.** 2008. A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. J. Immunol. **181**:4168–4176.
7. **Chen MI, et al.** 2010. 2009 influenza A (H1N1) seroconversion rates and risk factors among distinct adult cohorts in Singapore. JAMA **303**:1383–1391.
8. **Clark TW, et al.** 2009. Trial of 2009 influenza A (H1N1) monovalent MF59-adjuvanted vaccine. N. Engl. J. Med. **361**:2424–2435.
9. **Corti D, et al.** 2010. Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. J. Clin. Invest. **120**:1663–1673.
10. **Dormitzer PR, et al.** 2011. Influenza vaccine immunology. Immunol. Rev. **239**:167–177.
11. **El Bakkouri K, et al.** 2011. Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection. J. Immunol. **186**:1022–1031.
12. **Ellebedy AH, et al.** 2011. Impact of prior seasonal influenza vaccination and infection on pandemic A (H1N1) influenza virus replication in ferrets. Vaccine **29**:3335–3339.
13. **Ellebedy AH, et al.** 2010. Contemporary seasonal influenza A (H1N1) virus infection primes for a more robust response to split inactivated pandemic influenza A (H1N1) Virus vaccination in ferrets. Clin. Vaccine Immunol. **17**:1998–2006.
14. **Fang Y, et al.** 2010. Molecular characterization of in vivo adjuvant activity in ferrets vaccinated against influenza virus. J. Virol. **84**:8369–8388.
15. **Garten RJ, et al.** 2009. Antigenic and genetic characteristics of swine-origin 2009 A (H1N1) influenza viruses circulating in humans. Science **325**:197–201.
16. **Greenbaum JA, et al.** 2009. Pre-existing immunity against swine-origin H1N1 influenza viruses in the general human population. Proc. Natl. Acad. Sci. U. S. A. **106**:20365–20370.
17. **Guo H, Santiago F, Lambert K, Takimoto T, Topham DJ.** 2010. T

cell-mediated protection against lethal 2009 pandemic H1N1 influenza infection in a mouse model. J. Virol. **85**:448–455.
18. **Hancock K, et al.** 2009. Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. N. Engl. J. Med. **361**:1945–1952.
19. **Hillaire ML, et al.** 2011. Cross-protective immunity to influenza pH1N1 2009 viruses induced by seasonal A(H3N2) virus is mediated by virus-specific T cells. J. Gen. Virol. **92**:2339–2349.
20. **Ito R, et al.** 2003. Roles of anti-hemagglutinin IgA and IgG antibodies in different sites of the respiratory tract of vaccinated mice in preventing lethal influenza pneumonia. Vaccine **21**:2362–2371.
21. **Itoh Y, et al.** 2009. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature **460**:1021–1025.
22. **Janjua NZ, et al.** 2010. Seasonal vaccine effectiveness against pandemic A/H1N1. Lancet **375**:801–802.
23. **Kash JC, et al.** 2010. Prior infection with classical swine H1N1 influenza viruses is associated with protective immunity to the 2009 pandemic H1N1 virus. Influenza Other Respir. Viruses **4**:121–127.
24. **Kolaskar AS, Tongaonkar PC.** 1990. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett. **276**:172–174.
25. **Lamere MW, et al.** 2011. Contributions of antinucleoprotein IgG to heterosubtypic immunity against influenza virus. J. Immunol. **186**:4331–4339.
26. **Lamere MW, et al.** 2011. Regulation of antinucleoprotein IgG by systemic vaccination and its effect on influenza virus clearance. J. Virol. **85**:5027–5035.
27. **Laurie KL, et al.** 2010. Multiple infections with seasonal influenza A virus induce cross-protective immunity against A(H1N1) pandemic influenza virus in a ferret model. J. Infect. Dis. **202**:1011–1020.
28. **Liang XF, et al.** 2010. Safety and immunogenicity of 2009 pandemic influenza A H1N1 vaccines in China: a multicentre, double-blind, randomised, placebo-controlled trial. Lancet **375**:56–66.
29. **Lin J, Somanathan S, Roy S, Calcedo R, Wilson JM.** 2010. Lung homing CTLs and their proliferation ability are important correlates of vaccine protection against influenza. Vaccine **28**:5669–5675.
30. **Lin J, Zhi Y, Mays L, Wilson JM.** 2007. Vaccines based on novel adeno-associated virus vectors elicit aberrant $CD8^+$ T-cell responses in mice. J. Virol. **81**:11840–11849.
31. **Maines TR, et al.** 2009. Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. Science **325**:484–487.
32. **Miller E, et al.** 2010. Incidence of 2009 pandemic influenza A H1N1 infection in England: a cross-sectional serological study. Lancet **375**:1100–1108.
33. **Nguyen HH, van Ginkel FW, Vu HL, McGhee JR, Mestecky J.** 2001. Heterosubtypic immunity to influenza A virus infection requires B cells but not $CD8^+$ cytotoxic T lymphocytes. J. Infect. Dis. **183**:368–376.
34. **O'Neill E, Krauss SL, Riberdy JM, Webster RG, Woodland DL.** 2000. Heterologous protection against lethal A/HongKong/156/97 (H5N1) influenza virus infection in C57BL/6 mice. J. Gen. Virol. **81**:2689–2696.
35. **Rowe T, et al.** 2010. Modeling host responses in ferrets during A/California/07/2009 influenza infection. Virology **401**:257–265.
36. **Sambhara S, et al.** 2001. Heterosubtypic immunity against human influenza A viruses, including recently emerged avian H5 and H9 viruses, induced by FLU-ISCOM vaccine in mice requires both cytotoxic T-lymphocyte and macrophage function. Cell. Immunol. **211**:143–153.
37. **Sealy R, Surman S, Hurwitz JL, Coleclough C.** 2003. Antibody response to influenza infection of mice: different patterns for glycoprotein and nucleocapsid antigens. Immunology **108**:431–439.
38. **Skountzou I, et al.** 2010. Immunity to pre-1950 H1N1 influenza viruses confers cross-protection against the pandemic swine-origin 2009 A (H1N1) influenza virus. J. Immunol. **185**:1642–1649.
39. **Sun K, Ye J, Perez DR, Metzger DW.** 2011. Seasonal FluMist vaccination induces cross-reactive T cell immunity against H1N1 (2009) influenza and secondary bacterial infections. J. Immunol. **186**:987–993.
40. **Tu W, et al.** 2010. Cytotoxic T lymphocytes established by seasonal human influenza cross-react against 2009 pandemic H1N1 influenza virus. J. Virol. **84**:6527–6535.
41. **Waffarn EE, Baumgarth N.** 2011. Protective B cell responses to flu–no fluke! J. Immunol. **186**:3823–3829.
42. **Wang TT, et al.** 2010. Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. Proc. Natl. Acad. Sci. U. S. A. **107**:18979–18984.
43. **Wang W, et al.** 2011. Cross-neutralizing antibodies to pandemic 2009 H1N1 and recent seasonal H1N1 influenza A strains influenced by a mutation in hemagglutinin subunit 2. PLoS Pathog. **7**:e1002081.